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Flash-Induced Kinetic Infrared Spectroscopy Applied to Biochemical Systems

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Abstract. A flash photolysis apparatus with monitoring infrared beam is described allowing measurements of relative transmission changes of 10^{-3} in times of a few milliseconds. The investigation of the photodissociation of CO-myoglobin confirms the results obtained by static infrared difference spectroscopy. The application of our method to the rhodopsin/Meta II transition reveals signals which can tentatively be ascribed to the disappearance of the C=C-band of the protonated N-retinylidene Schiff base in rhodopsin. The developed method will be compared with other existing methods of kinetic vibronic spectroscopy such as kinetic resonance Raman spectroscopy and kinetic Fourier infrared spectroscopy.

Key words: Kinetic infrared spectroscopy – CO-myoglobin – Rhodopsin.

Fast kinetic spectroscopy has widely been employed for the study of chemical or biochemical reactions (e.g., flash photolysis, pulse, radiolysis, temperature jump), (Porter, 1963; Czerlinski and Eigen, 1959; Matheson and Dorfman, 1969; Rüppel and Witt, 1969). The measuring beam monitoring the reaction partners usually covers a spectral range from the near infrared to the ultraviolet; therefore, only electronic states of the reactants can be measured. A necessary prerequisite is that at least one reactant has a chromophore which absorbs strongly in this spectral range. In many cases changes in the electronic spectra are not easily interpretable at the molecular level. Therefore, supplementary methods are required to elucidate reaction mechanisms, e.g., Raman or infrared spectroscopy. Hitherto, however, only stable or long-lived products could be measured. The nature of transients often remains obscure. Almost all molecules have strong absorption bands in the mid-infrared, and molecular changes are often more easily interpreted on the basis of vibronic spectra. Thus, a valuable extension of kinetic spectroscopy would be kinetic infrared or Raman spectroscopy.

Since conventional Raman scattering is very weak and resonance Raman scattering is limited by disturbances produced by the probing beam and restricted by the necessary prerequisite of a chromophore, we decided to develop a method for kinetic spectroscopy in the mid-infrared spectral range.

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As a test for our method we used the photodissociation of carbon monoxide from myoglobin. This system was chosen for the following reasons: 1. The CO-binding to myoglobin has been investigated by conventional infrared-spectroscopy (McCoy and Caughey, 1971; Maxwell et al., 1974). 2. CO dissolved in water shows almost no absorption in the infrared due to extreme broadening of the CO-band; therefore, the photolysis of CO-myoglobin measured in the region of its CO-vibration should only show the spectrum of bound CO. 3. There are many investigations on the kinetics of the photodissociation of CO-myoglobin (Izuka et al., 1974; Austin et al., 1975). 4. The protein does not contribute to the absorption in the spectral range of the CO-band. It is expected, therefore, that the kinetic signals obtained at the wavelengths of the CO-band reflect changes of the CO-molecule only. 5. The background absorption caused by water is low, ensuring a high intensity of the infrared beam at the detector. 6. The CO-band cannot be seen in resonance Raman experiments, since the electrons of the heme chromophore do not couple to the vibrations of the CO-molecule.

In addition, we have applied our method to the rhodopsin/Meta II transition of the visual pigment of bovine rod outer segments. Since spectral changes are expected to occur in the spectral range between 1,800 cm^{-1} and 900 cm^{-1}, where all the constituents of the sample, i.e., proteins, lipids, water, chromophore, absorb strongly, the system is more complicated. Initial measurements of the static infrared difference spectrum rhodopsin vs. Meta II have shown that the transmission of the sample varies irregularly in regard to time, producing intensity fluctuations for times longer than several seconds. The fluctuation appeared to be larger than any spectral changes produced by bleaching rhodopsin. From these experiments we can deduce that, even if rhodopsin is completely bleached, the light induced transmission changes $\Delta I_\infty/I$ must be smaller than 5×10^{-2}. This imposes a requirement on the kinetic infrared apparatus: it must be able to resolve relative transmission changes smaller than 10^{-2} in times of a few milliseconds. As a guide as to where spectral changes caused by the chromophore can be expected, we use the resonance Raman data of rhodopsin (Mathies et al., 1976).

Description of the Apparatus and Materials

A schematic diagram of our apparatus is shown in Fig. 1. It resembles a conventional flash photolysis instrument. Radiation from a globar infrared source is modulated with a high speed chopper wheel at a modulation frequency of about 5 kHz. It passes through a filter Fi 1 which cuts off the visible part of the spectrum to prevent the sample from bleaching. After having passed the sample, the beam is dispersed by the monochromator, which is part of a Perkin-Elmer Infrared Spectrophotometer, and focussed onto a HgCdTe-detector from Santa Barbara Research Centers. The detector signal is amplified, filtered, and demodulated by a boxcar integrator. The reference signal is generated by the chopper wheel. The signal output of the demodulator is stored in a transient recorder, where signal averaging can also be performed if necessary. The sample is excited by a flash from which a filter Fi 2 cuts off light with wavelengths shorter than 530 nm in the case of rod outer segments, and wavelengths shorter than 380 nm in the case of CO-myoglobin. The chopping of the infrared beam proved to be necessary for measurements with time constants larger than one millisecond, since the HgCdTe-detector exhibits a 1/f-noise, dominating in the frequency range below about

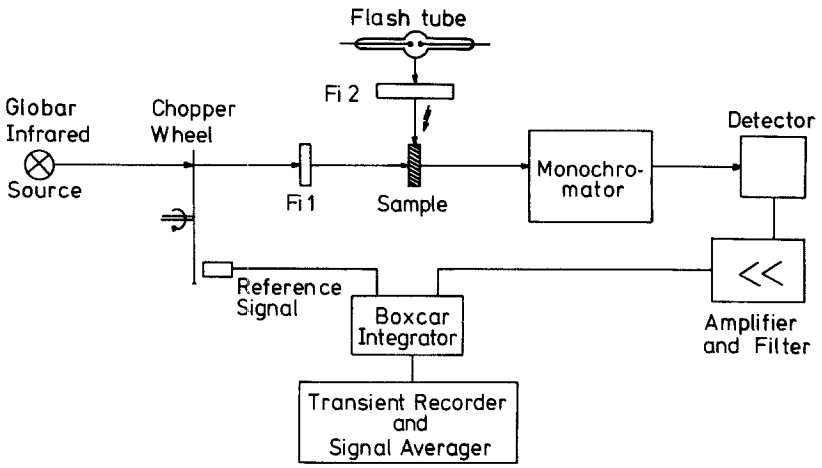

Fig. 1. Schematic diagram of the flash photolysis apparatus with infrared monitoring beam. Fi 1 and Fi 2 are optical filters for the infrared beam and exciting flash, respectively

2 kHz. The employment of such a detector, however, is necessary since thermopiles, normally used in infrared spectrophotometers are not only too slow but also not sensitive enough.

Sperm whale myoglobin was from Sigma Chemie München (type II). Samples were prepared at a concentration of 34 mg/ml in 0.1 M phosphate buffer pH 7.4. Myoglobin was reduced by approximately a tenfold excess of sodium dithionite. CO-myoglobin was produced by blowing CO for one hour on the surface of the myoglobin solution. Excess CO, dissolved in the buffer, was removed by blowing N_2 for one hour on the surface. CO-binding was controlled by recording the spectrum in the visible spectral region. Visible spectra, infrared spectra and kinetic signals were obtained from a sample of 25 μm thickness between two CaF_2-windows.

Rod outer segments from bovine retinae were prepared as previously described (Siebert et al., 1977). They are characterized by an absorbance ratio $A_{280/498}$ of 2.3 to 2.4, measured in 1% sodiumdeoxicholate. The rod outer segment suspension was sedimented in a small polyethylene tube (4 mm ∅), the tube was frozen in liquid nitrogen and but slightly below the interface between sediment and supernatant. The sediment was pressed out on a CaF_2-window and squeezed by a second window to a spacing of 12 μ. By this method, the water content of the sediment could be reduced, allowing measurements outside the water absorption bands at 3,400 cm^{-1} and 1,650 cm^{-1}. The infrared samples were tested for correct functioning by conventional UV-VIS-spectroscopy and flash photolysis.

Test Measurements and Discussion

Photodissociation of CO-Myoglobin

In Figure 2c we show the normal infrared spectrum of CO bound to myoglobin (solid line). It is in accordance with measurements by McCoy and Caughey (1971). Figure 2a

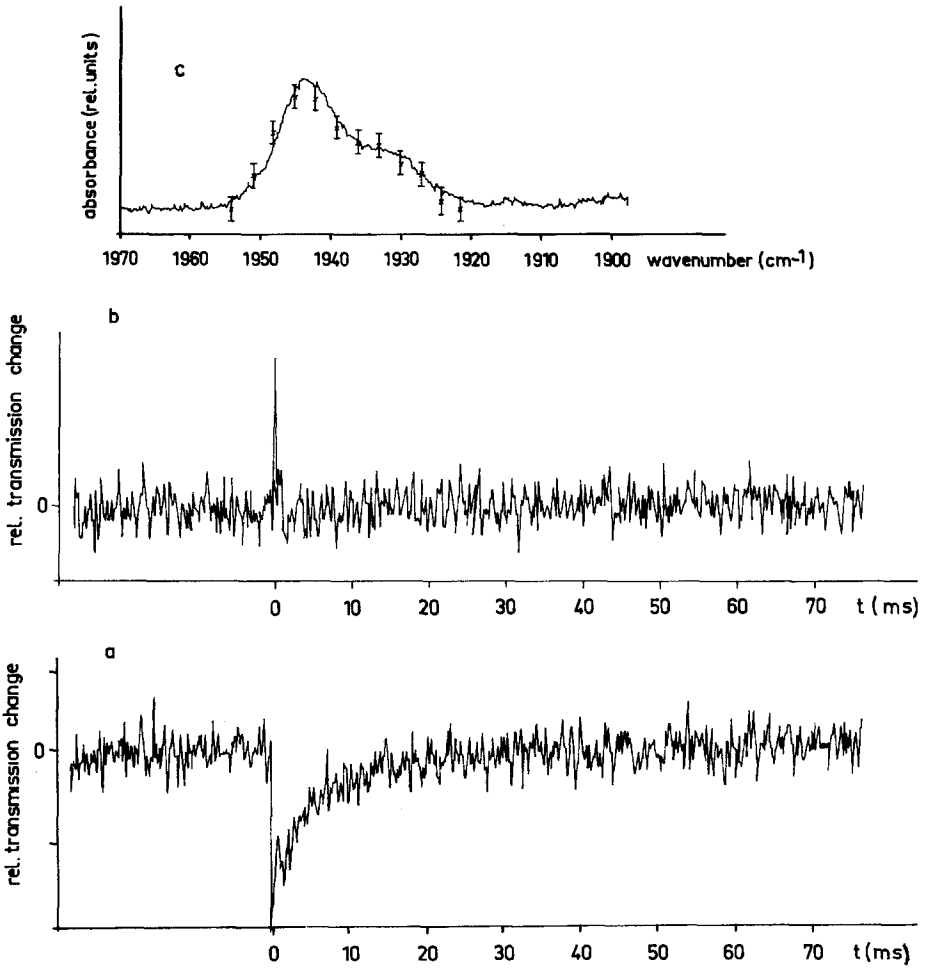

Fig. 2. CO-binding and photodissociation of myoglobin. **a** Photodissociation and rebinding of CO-myoglobin measured at 1,945 cm^{-1}. Temperature was 8° C to slow down the rebinding. The sample was photolysed with a flash of 100 μs duration. A filter cuts off wavelengths shorter than 380 nm. Approx. 40% of the CO-myoglobin was photolysed, which produced a transmission change of 5×10^{-3}. 100 signals were averaged to obtain the shown signal. Bandwidth was 3 cm^{-1}. **b** Baseline measured at 1,954 cm^{-1} to demonstrate flash artefacts. Other conditions were as in a. **c** Static infrared difference spectrum of CO-myoglobin vs myoglobin. 20 spectra were averaged (solid line). The data points with error bars are obtained from the normalized amplitudes of the kinetic signals at the corresponding wavelengths

shows a kinetic signal measured at 1,945 cm^{-1}, corresponding to the peak of the CO-band. It clearly demonstrates the dissociation and rebinding of CO. The time constant of rebinding, about 7 ms at 8° C, is in agreement with data given by Austin et al. (1975). As a control for flash artefacts we made a baseline measurement at 1,954 cm^{-1}, where no CO-absorption is observed (Fig. 2b). This shows that the flash artefacts are shorter than 300 μs. The data points with error bars in Fig. 2c, which are superimposed on the normal infrared spectrum, represent the normalized amplitudes of the kinetic signals at the respective wavenumbers.

The spectrum obtained by the kinetic signals reproduces the static spectrum. It has been argued that the shoulder around 1,933 cm^{-1} reflects a second binding site for CO in myoglobin. One could expect that for the second binding site a different quantum efficiency for photodissociation or a different time constant for rebinding should be observed. Since no spectral dependence of the time constant for rebinding has been observed, and since good agreement with the static spectrum has been obtained, our data do not provide evidence for a second binding site. We prefer the explanation that a fast equilibrium exists between two types of binding at the same binding site.

To resolve the shoulder at 1,933 cm^{-1}, the spectral bandwidth was reduced to 3 cm^{-1}. This resulted in a poor signal to noise ratio for a single signal. The trace shown in Fig. 2a was obtained by averaging 100 signals. By increasing the bandwidth to 9 cm^{-1}, resulting in a ninefold intensity, almost the same trace was obtained for a single signal. (For the infrared intensities employed, the HgCdTe-detector does not show an intensity dependent noise.)

Our results show that additional information can be obtained by the method of flash-induced kinetic infrared spectroscopy: by conventional flash photolysis the kinetics of the rebinding of CO are obtained, but only limited information about the mode of binding is provided. On the other hand, static infrared spectroscopy illuminates the mode of binding, but lacks the information on the kinetics. By extending the investigations to lower temperatures and by improving the time resolution, intermediate stages of the rebinding can perhaps be detected as they have been postulated from conventional flash photolysis experiments at low temperatures (Austin et al., 1975).

Rhodopsin/Meta II Transition

In Figure 3 a signal is shown representing the flash induced transmission change of a rod outer segment sample measured at 1,555 cm^{-1}. This wavenumber has been chosen, as a strong absorption band can be expected from resonance Raman measurements on rhodopsin (Mathies et al., 1976) and from infrared data on model compounds (Blatz and Mohler, 1975). This band can be ascribed to the C=C-vibration of the retinal. We made additional measurements at 1,600 cm^{-1} and at 1,540 cm^{-1}, where, however, no transmission changes could be detected. Thus, the light-induced transmission changes exhibit a strong spectral dependence, excluding the presence of unspecific artefacts. The time constant for the transmission increase in Fig. 3 is approximately 10 ms, indicating that in fact the rhodopsin/Meta II transition has been observed. The transmission increase can be explained by a shift of the C=C-band of the retinal in rhodopsin to a higher wavenumber in Meta II (Doukas et al., 1978). The complete kinetic infrared rhodopsin/Meta II difference spectrum between 1,800 cm^{-1} and 1,100 cm^{-1} will be presented in a subsequent paper (Siebert et al., 1980).

This example shows, that flash induced kinetic infrared spectroscopy can yield detailed results even in the presence of the large continuum absorption of the water and in the presence of the large absorption caused by the amide II-band. As mentioned earlier, it was not possible to obtain a static infrared rhodopsin/Meta II difference spectrum.

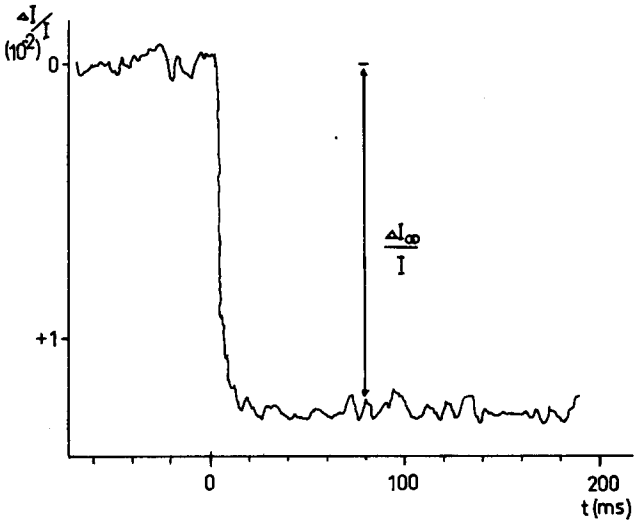

Fig. 3. Flash induced signal of a rod outer segment sample measured at 1555 cm^{-1}. Approximately 20% of the rhodopsin was bleached by the flash from which light with wavelengths shorter than 530 nm was cut off by a filter. Flash duration was approx. 1 ms. Sample temperature was 20° C. $\Delta I_i \infty/I$ represents the final relative transmission change

General Discussion

We have developed a method of kinetic infrared spectroscopy by extending the conventional flash photolysis into the infrared spectral range. Our method is especially suited for the detection of small spectral changes. Relative transmission changes of 10^{-3} can be detected in a few milliseconds. This method has been successfully applied to two biochemical systems. In both cases the high sensitivity was essential. This is a prerequisite for most biochemical systems, since they are composed of many subunits contributing to the background absorption. Only a small part of the system will be directly involved in the biological function, thereby exhibiting only small spectral changes.

At present our method suffers from limited time resolution. This is due to the mechanical chopper which limits the modulation frequency to 10 kHz. Higher frequencies could be obtained by an electro-optic-modulator. In our experiments, the signal to noise ratio is limited by mechanical inaccuracies of the chopper wheel. If an ideal modulation were used, the signal to noise ratio could be increased by a factor of ten. For reactions faster than 10 μs no modulation would be necessary, since the 1/f-noise could be eliminated by a high-pass filter. Then, however, the low intensity of the infrared beam would limit the time resolution.

In the past several methods of kinetic infrared spectroscopy have been applied (a survey is given by Pimental, 1968). In these cases a very fast scanning dispersive element is used to scan the spectral region several times during the kinetic process. This method is limited to investigations where large spectral changes are to be measured. Another approach to kinetic infrared measurements is the application of Fourier transform spectroscopy (a survey is given by Durana and Mantz, 1978).

A possible adaptation of the Fourier spectroscopy to flash-induced infrared spectroscopy would be the following set up: At each stepping point of the interferometer a flash would be applied to the sample producing a corresponding intensity change at the detector. The flash-induced time dependent signals would be digitized and stored in a memory. By this method a time dependent interferogram would be obtained. Performing the Fourier transform for each time of interest, the corresponding flash-induced infrared spectrum at this time would be obtained. It is evident that for this method a large mass storage is required. In addition, since inaccuracies in the amplitude of the interferogram limits the spectral resolution, very reproducible transients are needed. This confines the use of Fourier transform spectroscopy to either reversible processes or to samples which can be reproduced quickly with high accuracy. If these conditions are met, the multiplex advantage and the higher energy available in Fourier transform spectroscopy reduce the total measuring time considerably.

Recently several papers on kinetic resonance Raman spectroscopy applied to bacteriorhodopsin were published (Campion et al., 1977a, b; Terner et al., 1977; Marcus and Lewis, 1977). Notwithstanding, two properties of the resonance Raman spectroscopy should be taken into account if applied to kinetic studies: 1. The measuring beam, its wavelength being near an absorption band, could perturb the sample. 2. Only vibrations belonging to the chromophore are observable. These difficulties do not exist in kinetic infrared spectroscopy. With this method, however, only differences of states can be detected, if complex systems, such as proteins with a chromophore, are investigated. The accuracy of normal infrared spectra does not suffice to detect details, e.g., the chromophore in rhodopsin. Therefore, the interpretation of the difference spectra requires supplementary methods such as resonance Raman spectroscopy. Both methods of vibronic spectroscopy appear to be very useful for the study of chemical or biochemical reactions, and limitations imposed by one method are, at least to some extent, eliminated by the other.

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